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**Progress Report on Grant N00014-91-J-1217**

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**GRANT TITLE:** In Vivo Distribution of Liposome Encapsulated Hemoglobin Studied With Imaging Radiotracers

**START DATE:** 12/01/90

**RESEARCH OBJECTIVE:** This project has as its objective the development of radiotracer imaging technology to follow the in vivo circulation and organ deposition of liposome encapsulated hemoglobin (LEH). LEH will be labeled with technetium-99m ($^{99m}$Tc) or indium-111 ($^{111}$In) and infused into small animals to monitor any in vivo differences between different LEH formulations. These studies will be correlated with any hematological and pathological changes associated with LEH treatment. Development of such non-invasive monitoring techniques may lead to significant cost effective manufacturing and formulation improvements, and ultimately a more efficacious LEH product. The development of this elegant labeling technique should make it possible to study the effect of various LEH modifications on biodistribution non-invasively in primates and humans.

**PROGRESS:** Our research progress for the period of December 1, 1992 to April 1, 1993 is covered in this report. We have completed a study to determine if our imaging techniques could be used to quantitate the biodistribution of different resuscitative fluids in a hypovolemic rat model. Following removal of a known volume of blood equal to 50% of the blood volume by total body weight from the femoral artery catheter, we infused 2 ml of $^{99m}$Tc labeled red blood cells and watched the change in their circulation after replacing the additional volume with a resuscitative fluid. We have completed imaging studies for replacement with either normal saline (0.9%), hypertonic saline (7.5% at 5ml/kg), shed blood, LEH, or no resuscitative fluid. We have completed the image analysis for each test fluid. A region of interest was drawn around

various organs and the radioactivity associated with that region compared to the total body activity. Region to total body ratios were determined out to 90 minutes at 15 minute intervals following the infusion of labeled red blood cells. We are most interested in the increases or decreases of blood pool in the peripheral muscles and abdominal organs which are good indicators of the amount of oxygenation of these areas following resuscitation. We saw an increase in activity in the spleen in the animals receiving no resuscitation or saline groups except the shed blood group. The spleen activity in LEH group increased, but to a lesser degree than the shed blood group. There was a greater increase in activity in the liver in animals receiving no resuscitation, saline, and LEH than for the shed blood. There was no significant difference in any of the groups in the heart which represents the blood pool. In the abdomen, there was higher activity for replacement with shed blood and the saline groups over no resuscitation and LEH animals. We saw no significant difference in the proximal or distal periphery for any of the groups. Although these comparisons have been useful in determining the biodistribution pattern following hypovolemia, we feel this particular imaging technique is not sensitive enough to detect the subtle differences in the resuscitative fluids studied thus far. We also determined the changes in mean arterial pressure and heart rate following resuscitation for the various fluids. As expected the mean arterial pressure and heart rate of the shed blood group recovered to baseline values after reinfusion. Rats receiving no resuscitative fluid recovered and maintained pressure, but heart rate leveled off at 60 beats per minute less than baseline. The saline resuscitations fell between these two control fluids with the hypertonic saline group recovering faster and leveling off, while the 0.9% saline group had a decrease of 20 in mean arterial pressure and a decrease of 50 in heart rate. The mean arterial pressure and heart rate of the LEH group recovered to baseline levels by 90 minutes.

We completed a study to determine the clearance of $^{99m}$Tc-LEH in rats over a 72 hour period. This study was conducted to determine the clearance and metabolism of $^{99m}$Tc-LEH from the body, which is an important consideration in elucidating if there is any long-term toxicity associated with LEH administration. The animals (n=4 per group) were injected with $^{99m}$Tc-LEH (142 mg/kg phospholipid and 836 mg/kg hemoglobin of body weight in 2 ml) via the tail vein. At 1, 2, 4, 24, 48, and 72 hours, the animals were sacrificed. Tissues were weighed and counted for radioactivity. The $^{99m}$Tc-LEH decreased in the bloodstream from 46.1% at 1 hour to 20.9% at 4 hours and continued to be removed from

circulation with approximately 1% of the activity remaining at 24 hours. The major portion of the activity was located in the RES organs of the liver, spleen and bone marrow. The activity in the kidney remained at 2% and was relatively constant over the 72 hour period. Bowel activity was constant out to 24 hours and dropped gradually out to 72 hours. Large increases were noted in the urine and feces with time. The urine activity rose from 2.2% at 4 hours to 13.9% at 24 hours and continued to increase to 28.2% at 72 hours post-injection. Feces activity showed a similar clearance profile as urine, but the large increase in feces activity did not occur until 48 hours after injection of the $^{99m}$Tc-LEH. The muscle, skin, brain and lungs played only a minor role in the clearance of the $^{99m}$Tc-LEH.

We have begun a study to determine the changes in platelet biodistribution as a function of LEH administration. This study was proposed because of the evidence by Dr. Reuven Rabinovici that certain batches of LEH caused thrombocytopenia and increased production of Thromboxane B2. We decided to use an imaging protocol to try and follow the distribution of labeled platelets following an infusion of LEH. The labeling of platelets with indium-111 from both rats and rabbits has been set up in our laboratory. The animals were reinfused with autologous labeled platelets and monitored under a gamma camera. Following a ten minute equilibration period, an injection of LEH was given intravenously. The biodistribution of the labeled platelets changed dramatically with the platelets leaving the blood pool and localizing in the lungs. This effect was transient with the return of platelets to the blood pool occurring within 30 minutes. A similar phenomenon has been seen with liposomes containing no hemoglobin, but having a negative surface charge.

**WORK PLAN**: During the next funding period, we will continue to use our $^{99m}$Tc liposome labeling protocol to test LEH formulations as supplied by NRL or Vestar for their circulation properties and organ distribution. A LEH formulation being developed by Vestar which can be produced at a smaller more homogeneous size for sterile filtration has been modified for scale up. Biodistribution studies with these new LEH preparations that contain recombinant human hemoglobin will be studied as soon as these preparations become available.

Although our $^{99m}$Tc labeling procedure has provided valuable data concerning the biodistribution of LEH, it does not allow us to follow the ultimate metabolic fate of the hemoglobin. This information is very important for the safety of LEH as a blood substitute since we want a product which will be cleared from the body and produce few toxic side

effects. To study this problem, we plan to label hemoglobin with $^{3}H$ and $^{14}C$ using a mild reductive methylation procedure. This mild labeling technique has been used to label the lysine residues of a number of proteins including hemoglobin without affecting the functionality of the protein. The hemoglobin will be supplied by NRL. Also the radiolabeled starting material used in the procedure is available from commercial sources. Once labeled, the hemoglobin will be used to make LEH. The labeled LEH will then be double labeled using the $^{99m}Tc$ liposome labeling protocol. This double labeled material will be injected into animals and imaged under the gamma camera. The animals will then be sacrificed for tissue biodistribution measurements. Samples of the tissues will be counted for both gamma activity as well as for $^{3}H$ or $^{14}C$ using liquid scintillation counting. This study will provide important information concerning the fate of both the hemoglobin and liposomal components of LEH.

We also plan to study the efficacy of LEH using a positron emitting isotope of oxygen ($^{15}O$). To our knowledge, this study will be the first attempt to actually quantitate and image oxygen delivery to tissues. These studies are uniquely possible at our institution because of our newly operational cyclotron and positron emission tomography (PET) camera located at our Research Imaging Center and the previous experience of our group in both imaging and blood substitutes. The biodistribution studies using $^{99m}Tc$ have been very useful, but do not provide any information about how efficient LEH is in delivering oxygen to the tissues in vivo. We will attempt to develop a PET imaging protocol to quantitatively determine the amount of oxygen extracted by the brain, liver and skeletal muscle in rabbits given $^{15}O$ labeled LEH. The oxygen extraction fraction will be compared to control animals receiving $^{15}O$ labeled blood. This technique will then be applied to animals subjected to a 50% blood withdrawal and resuscitation.

We also plan to test our $^{99m}Tc$ labeled liposomes as an imaging agent for the detection of atherosclerotic disease in collaboration with Dr. Bailey at our institution. Such an agent could be used not only to screen patients with the disease, but also to follow the efficacy of cholesterol lowering drugs in the treatment of atherosclerosis.

We plan to continue our platelet distribution studies. We plan to determine any differences in this transient platelet redistribution following infusion of liposomes containing no hemoglobin or hemoglobin alone.

**INVENTIONS:** The European patent rights for the $^{99m}$Tc liposome labeling procedure are being filed. A licensing agreement between Lipotek INC and The University of Texas Health Science Center at San Antonio/Department of the Navy is under negotiation.

**PUBLICATIONS AND REPORTS:** The manuscript to Critical Care Medicine with Dr. Rudolph at NRL describing our results of the circulation persistence and biodistribution of lyophilized LEH was accepted. We have submitted a manuscript outlining our results using $^{99m}$Tc labeled liposomes in infection imaging to Journal of Nuclear Medicine
We presented a report entitled " Labeling red blood cells with copper-67" at the Southwest Chapter of the Society of Nuclear Medicine meeting in Dallas, Texas on March 11-14, 1993. We presented a poster entitled "Circulation profile of technetium-99m labeled liposome encapsulated hemoglobin in a 10% or 50% rat hypovolemic shock model" at the V$^{th}$ International Symposium on Blood Substitutes held in San Diego, California on March 17-20, 1993. We are currently preparing a manuscript describing our results using $^{99m}$Tc labeled liposomes in a tumor model. Also we plan to complete a manuscript describing the circulation kinetics and biodistribution of $^{99m}$Tc labeled LEH in a rat hypovolemic model.

**TRAINING ACTIVITIES:** None